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Inhibition of cyclooxygenase-2 expression by zinc-chelator in retinal ischemia

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Abstract

The zinc ion (Zn^{2+}) is abundant in neurons. However, excessive Zn^{2+} can induce neuronal cell death. This study examined the role of Zn^{2+} in transient retinal ischemia in adult male rats. The rats were sacrificed 4–24 h after retinal ischemia by high intra-ocular pressure, and the retinas were prepared for microscopic examination of retinal cell degeneration, and fluorescence microscopy using zinquin ethyl ester as the zinc ion-specific probe. Moreover, COX-2 expression was observed by Western blotting. In control retinas, there was a low Zn^{2+} concentration in the inner plexiform layer (IPL), a high Zn^{2+} concentration in the outer plexiform layer (OPL), and no detectable Zn^{2+} in either the ganglion cell layer (GCL) or the inner nuclear layer (INL). In contrast, in the retinas exposed to ischemia without the administration of the zinc ion chelators (Ca^{2+}-EDTA and TPEN), Zn^{2+} deposits were found in the IPL and INL beginning 4 h after ischemia and degeneration of neurons was found in the GCL and INL. Less Zn^{2+} accumulation in the IPL and INL and less neuronal degeneration in the GCL and INL were found in the retinas treated with Ca^{2+}-EDTA or TPEN before ischemia. Furthermore, the COX-2 protein levels increased 4–8 h after retinal ischemia, and chelation of zinc ion inhibited this effect. These results suggest that the accumulation of Zn^{2+} following an ischemic insult can cause retinal degeneration and induce abnormal COX-2 expression.

Abbreviations: Ca^{2+}-EDTA, ethylenediaminetetraacetic acid saturated with equimolar Ca^{2+}; TPEN, N,N,N′,N′ tetrakis-(2 pyridylmethyl) ethylenediamine; COX-2, cyclooxygenase 2

Keywords: Retina; Ischemia; Zinc ions; Zinquin ethyl ester; Ca^{2+}-EDTA; TPEN; COX-2

1. Introduction

Retinal ischemia induces neuronal degeneration in the ganglion cell and inner nuclear layers and degeneration of one or more types of retinal neurons leads to the loss of vision. Ischemic retinopathy can cause the degeneration of retinal neurons in glaucoma and diabetic retinopathy (Levin, 2001).

In diabetic retinopathy, the loss of vision is due to degeneration of the retinal neurons a result of a blockage of the blood supply. In glaucoma, the high-pressure in the eyeball also induces retinal ischemia and glutamate-related retinal toxicity (Osborne et al., 2004). Neuronal cell degeneration in retina and brain after ischemic injury involves free radicalproductions, glutamate excitotoxicity, and influx of calcium and zinc (Choi, Weiss, Koh, Christine, & Kurth, 1989; Clemens, 2000; Joo et al., 1999).

Zinc ion (Zn^{2+}) is essential for the formation, activation, and regulation of the zinc metalloenzymes, superoxide dismutase (SOD), and zinc finger protein (Frederickson, Suh, Silva, & Thompson, 2000). Zinc ion modulates of neural transmission and is reported to affect gamma aminobutyric acid (GABA), glycine, and NMDA receptor channels

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(Kaneda, Andrasfalvy, & Kaneko, 2000; Qian, Li, Chappell, & Ripps, 1997). It is present in the axon terminals of the glutamatergic neurons in the central nervous system (Franco-Pons et al., 2000) and it is abundant in the inner segment of the photoreceptor cells of the retina and in retinal pigment epithelium and at low concentrations in the ganglion cell layer (GCL) and inner nuclear layer (INL) (Akagi, Kaneda, Ishii, & Hashikawa, 2001; Grahn, Paterson, Gottschall-Pass, & Zhang, 2001; Ugarte & Osborne, 2001). Furthermore, zinc ions are actively controlled by the pigment epithelium, and exogenous zinc ions increase the catalase activity. Zn^{2+} concentration is very important for metabolism in the retinal pigment epithelium, where it plays a role in protection from oxidative stress (Miceli, Tate, Alcock, & Newsome, 1999; Newsome, Oliver, Deupree, Miceli, & Diamond, 1992).

However, zinc ion has been implicated in excitotoxic effect in the central nervous system, and plays a role in neuronal degeneration. In addition, zinc ion induce cell death in neural cell cultures and has been found to accumulate in neurons after brain ischemia (Canzoniero, Manzerra, Sheline, & Choi, 2003; Kim, Kim, Gwag, Sohn, & Koh, 1999; Marin, Israel, Glowinski, & Premont, 2000; Weiss, Sensi, & Koh, 2000). It also accumulates in the ischemic retina and inhibits energy production (Yoo, Lee, Lee, Koh, & Yoon, 2004).

There are three isoforms of cyclooxygenase (COX), COX-1, COX-2, and COX-3. COX-1 is expressed constitutively in most cells while COX-2 is only induced under abnormal conditions. COX-3 is an alternatively spliced form of COX-1 whose function is unclear. COX-2 is a pro-inflammatory enzyme that is expressed after ischemic injury to the brain and retina. COX-2 is known to be an early response enzyme in neural ischemia (Dash, Mach, & Moore, 2000; Hewett, Uliasz, Vidwans, & Hewett, 2000) and its overexpression rapidly induces cell death (Dore et al., 2003). Conversely, inhibition of COX-2 protects neurons from neuronal cell death and inflammation (Ju, Kim, & Neufeld, 2003; Sasaki et al., 2004).

The aim of this study was to examine the relationship between the Zn^{2+} translocation and COX-2 expression after retinal ischemia. We also assessed observed the effect of inhibiting Zn^{2+} translocation on ischemia-induced retinal degeneration.

2. Materials and methods

2.1. Experimental animals

The subjects for this study were Sprague–Dawley rats (male, 200–250 g each). All experiments adhered to the Principals of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and Institutional Animal Care and Use Committee of the Catholic University of Korea. Rats were housed under standard condition and anesthetized with xylazine (20 mg/kg, Yuhan corp., Seoul, Korea). Retinal ischemia was induced in the left eye of rats by application of high intra-ocular pressure (160 mmHg) for 1 h. The high intra-ocular pressure was induced in anterior chamber by needle (30 guage, Sigma, St. Louis, MO, USA) while observing a pressure meter. The right eyes were used as non-ischemic controls after anesthetization.

2.2. Degeneration of retinal neurons and labeling of RGCs

The eyes were fixed in 4% paraformaldehyde for 2 h. The retinas were embedded in paraffin after dehydration by alcohol. The paraffin-embedded retinal tissues were sectioned at 6 μm by microtome (Leica, Vienna, Austria). After de-paraffin, sectioned tissues were stained for nuclear condensation (pyknotic cells) with Hoechst 33258 (2 μg/ml in saline, Sigma) and examined under fluorescent microscopy (Carl Zeiss, Hallbergmoos, Germany). We selected 10 retinas in each group for cell count in the experimental retina. The number of cells was counted in a 500 × 100 μm square of the GCL and of the INL and expressed as a percentage in each of the five areas in selected from five different retinas from each group.

For counting of retinal ganglion cells (RGCs), 4Di-10ASP (Molecular Probe, Eugene, OR) was injected into the superior colliculus by the stereotaxic instrument with micro-injector (Stoelting, Wood dale, IL) at five days before ischemia. 24 h after ischemia, the rats were sacrificed and eyes were enucleated. The retinas were separated from the choroids and sclera, and flat-mounted on microscope slides. The labeled ganglion cells were counted in a 0.5 × 0.5 mm square under a fluorescence microscope (Carl Zeiss, Jena, Germany).

The number of cells was expressed as a percent of those seen in normal retina. The data are expressed as value (mean ± SEM). The statistical significance of differences in each group means was assessed using two-way analysis of variance (ANOVA) and Student's *t* test.

2.3. Chemical administration

As controls of ischemic experimental rats and non-ischemic control eyes, 10 μl (approximately one-third of vitreous volume) of 0.9% saline was injected 10 min before induction of ischemia. Zinc translocation was inhibited by Ca^{2+}-EDTA (1 mM, extracellular zinc chelator, Sigma) and TPEN (100 μM, *N*,*N*,*N*′,*N*′ tetrakis-(2 pyridylmethyl) ethylene diamine) (cell-permeable zinc chelator, Calbiochem, La Jolla, CA, USA). The Ca^{2+}-EDTA was dissolved in saline and the TPEN was dissolved in 10% DMSO. The zinc chelators (10 μl per eye) were injected into the vitreous body at 10 min before induction of ischemia. The experimented rats were 10 animals in each group. Rats were sacrificed 4–24 h after reperfusion by high dose anesthetization.

2.4. Zinc ion accumulation assay

We used zinquin ethyl ester (Calbiochem) for detection of intracellular zinc. After enucleating, the eyes were frozen on dry ice directly. The cryo-embedded retinas were sectioned at 10 μm, dried at room temperature, and stained with zinquin ethyl ester (10 μM, in DMSO) for 5 min at the dark chamber. After rinsing in 0.9% saline, sections were examined under the fluorescence microscope with a differential interference contrast (DIC) filter (Zeiss, Germany). The excitation and emission was 358 and 485 nm, respectively. We selected 10 retinas in each group for analysis.

2.5. Western blot analysis for COX-2 expression

Retinas separated from eyeballs on an iced plate and retinas were stored at −70 °C until use. The retinas were lysised for 10 min at 4 °C in 200 μl of lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin). After homogenizing and centrifugation, the supernatant was collected to total protein values. Twenty micrograms of protein from each sample were loaded on SDS–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA) at 350 mA for 70 min after methanol activation. Antibody reaction was performed after blocking with 5% skim milk. The membrane was incubated with polyclonal rabbit anti-COX-2 antibody (1:1000, Santa Cruz biotechnology, Santa Cruz, CA, USA) overnight at 4 °C and washed three times in PBST (PBS containing 0.1% Twin 20) buffer. And then the membrane was incubated with

horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Santa Cruz biotech.) for 1 h. After three times washes with PBST buffer, HRP activity was visualized on X-ray film (Fuji film, Tokyo, Japan) by Chemiluminiscence (ECL) (Western blotting luminol reagent (Santa Cruz biotech.)).

3. Results

3.1. Zinc accumulation after retinal ischemia

We have visualized the distribution of zinc with zinquin, and have found that the location of zinc in the retina changed after ischemia.

As shown in Fig. 1A, zinc was strongly concentrated in the inner segment of the photoreceptor cells in the non-ischemic control rats, and was not detected in either the ONL or the INL. However, accumulation of zinc was observed in both the IPL and INL beginning 4 h after ischemia and in GCL, zinc staining was stronger and more diffuse than in the non-ischemic controls (Fig. 1).

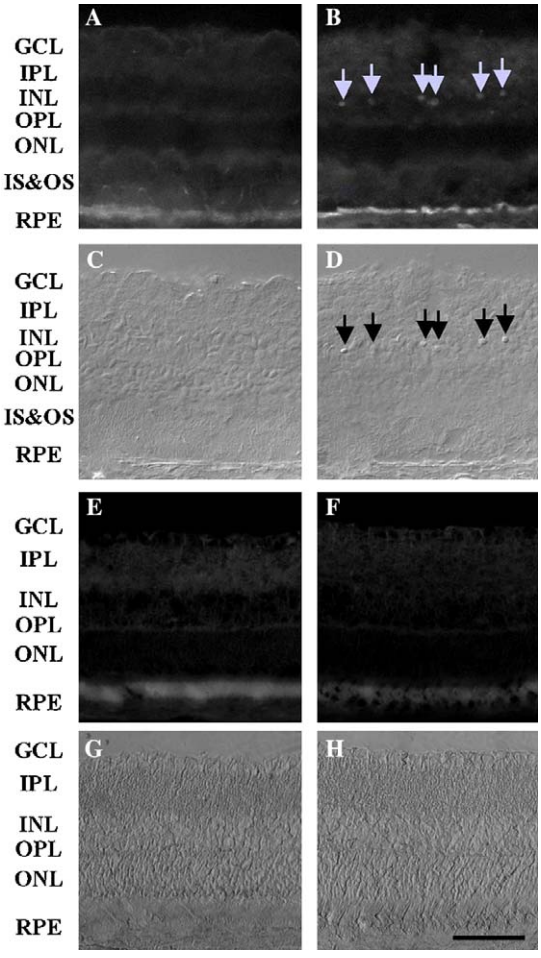

Fig. 1. Fluorescence microscopy of zinquin-stained Zn^+ reveals co-localization of areas of retinal cell death and increased Zn^+ concentrations. (A) Non-ischemic control retina. (B) Ischemic control retina 4 h after reperfusion. (C) DIC image of non-ischemic control retina. (D) DIC image showing the presence of pyknotic cells in an ischemic control retina 4 h after reperfusion. (E) Ca^{2+}-EDTA (1 mM) treated ischemic experimental retina 4 h after reperfusion. (F) TPEN (100 μM) treated ischemic experimental retina 4 h after reperfusion. (G) and (H) DIC images of the sections in (E) and (F), respectively. White arrows, Zn^+: dark arrows, pyknotic cells; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium. Scale bar = 100 μm.

The accumulation of zinc and the appearance of compact, round pyknotic cells occurred in at the same locations in the ischemic retinas (Figs. 1B and D). Pyknotic cells were detected in the INL using light microscopy with a DIC filter (Fig. 1D) and zinc was strongly concentrated in the same areas (Fig. 1B). However, no pyknotic cells were detected in the INL of the non-ischemic control retinas (Fig. 1C). The accumulation of zinc and pyknotic cells was inhibited by chelating zinc with 1 mM of Ca^{2+}-EDTA (Figs. 1E and G) or 100 μM TPEN before inducing ischemia (Figs. 1F and H). No effect of 10% DMSO was not detected on the retina.

3.2. Zinc chelation inhibits COX-2 expression

We also asked whether the accumulation of zinc induces COX-2 expression (Fig. 2). Western blots of COX-2 (approximately 74 kDa) were performed 4, 8, and 24 h after ischemia-reperfusion. Although low levels of COX-2 were observed in the normal retina and control experiments (Fig. 2E), COX-2 expression was increased from 4 h after ischemia (Fig. 2A). And Ca^{2+}-EDTA and TPEN inhibited this effect to similar extents (Fig. 2B). To confirm the role of zinc in COX-2 expression, we performed the experiments shown in Figs. 2C and D, in which $ZnCl_2$ (1 mM, 10 μl) was injected into vitreous body as control experiments. Injection of $ZnCl_2$ also induced COX-2 expression but induction was greater at 24 h than 8 h (Figs. 2B and D). COX-2 expression was unaffected by Ca^{2+}-EDTA and TPEN on their own (Figs. 2E and F).

3.3. Inhibition of retinal degeneration by zinc chelators

To examine the relationship between retinal cell death and zinc, we observed retinal cell death by staining with Hoechst-33258 and retrograde labeling. Fig. 3A shows normal retinal neurons in the GCL and INL of the non-ischemic control retinas, and Fig. 3B shows degenerated retinal neurons in the GCL and INL 24 h after ischemia from the ischemic control retinas. Cells with pyknotic nuclei in the GCL and INL are indicated by arrowheads in Fig. 3B. Ca^{2+}-EDTA (Fig. 3C) and TPEN (Fig. 3D) reduced retinal degeneration in the GCL and INL 24 h after ischemia. In ischemic retinas not injected with zinc chelators, 60 ± 7% of cells in the GCL and 47 ± 5.2% of cells in the INL had degenerated 24 h after reperfusion. With Ca^{2+}-EDTA (1 mM) pretreatment, only 17 ± 5% of cells in the GCL and 21 ± 5.5% of those in the INL had degenerated, and with TPEN (100 μM) pretreatment, 22 ± 7.5% of cells in the GCL and 18 ± 5% of those in the INL had degenerated (Fig. 3E). Retrograde labeling of retinal ganglion cells (RGCs) was performed to confirm the protective effect of zinc chelators in the GCL (Fig. 4). In ischemic retina in the

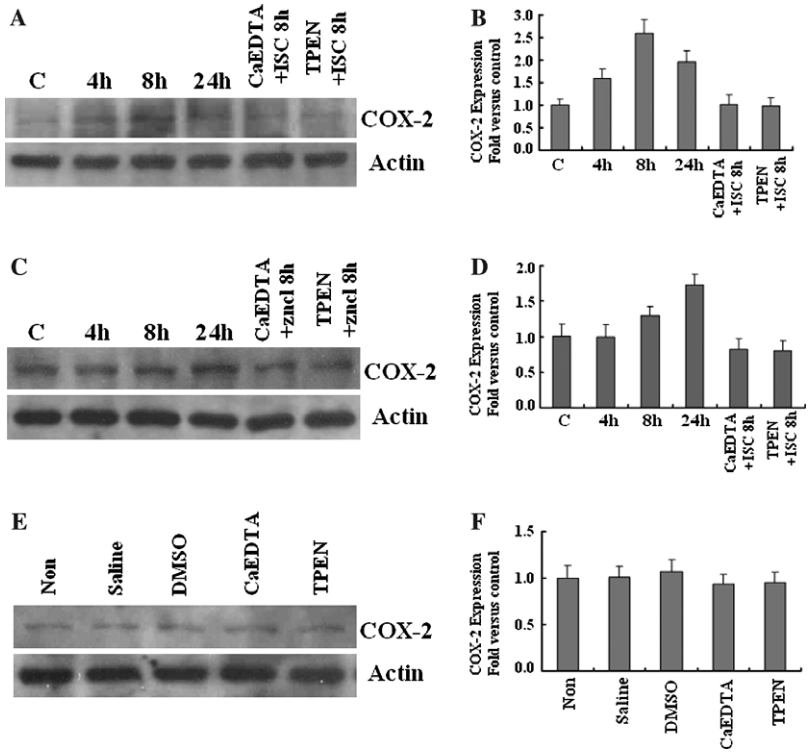

Fig. 2. Expression of COX-2 in ischemic retinas. Expression of COX-2 in response to ischemia (A and B) and exogenous $ZnCl_2$ (C and D), with and without prior treated of 1 mM Ca^{2+}-EDTA and 100 μM TPEN. (E and F) The data show control experiment. Each reagent did not induce COX-2 expression 8 h after treatment in normal retina. The data for each column in (B), (D), and (F) were taken from three different experiments. The quantitative data were obtained by densitometrc analysis, and are means ± standard error (SEM).

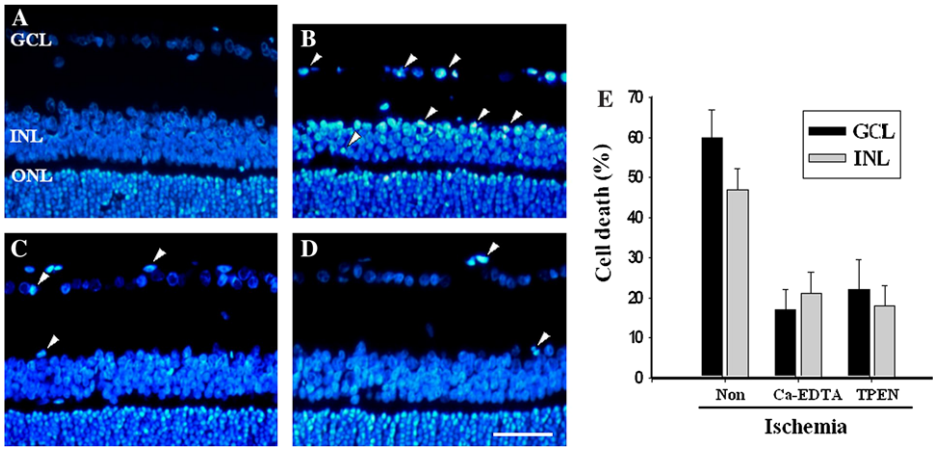

Fig. 3. Hoechst staining reveals retinal cell degeneration 24 h after ischemia. (A) A non-ischemic retina; (B) is an ischemic control retina; (C) is an ischemic retina pretreated with Ca^{2+}-EDTA; (D) is ischemic experimental retina pretreated with TPEN. Arrowheads indicate cells with pyknotic nuclei. Scale bar = 50 μm. The protective effect of Zn^{+} chelator is shown in the form of quantitative data in (E) which are means ± standard errors (SEM) of the numbers of cells remaining in the retina per 500 × 100 μm area 24 h after experimental ischemia with/without zinc chelators. Cell death (%) in the Zn^{+} chelator treated groups was significantly lower than in the non-treated group ($p < 0.05$ by ANOVAs).

absence chelators (Fig. 4B), 46 ± 3.5% of the control number of RGCs were observed, and this number increased to 77 ± 4.5% in the Ca^{2+}-EDTA treated group (Fig. 4C) and to 74 ± 5% in the TPEN treated group (Fig. 4D). The quantitative data are given in Fig. 4E. In normal retinas, there number were 687 ± 21 RGCs per 0.5 mm^2, and the chelators significantly inhibited retinal degeneration, ($p < 0.05$, vs. ischemia without chelators). ANOVA and Student's t test showed that there was no difference between the protected effects of Ca^{2+}-EDTA and TPEN.

4. Discussion

Neural ischemia raises glutamate level and activates NMDA and kainate/AMPA receptors. High pressure-induced retinal ischemia also causes release of glutamate

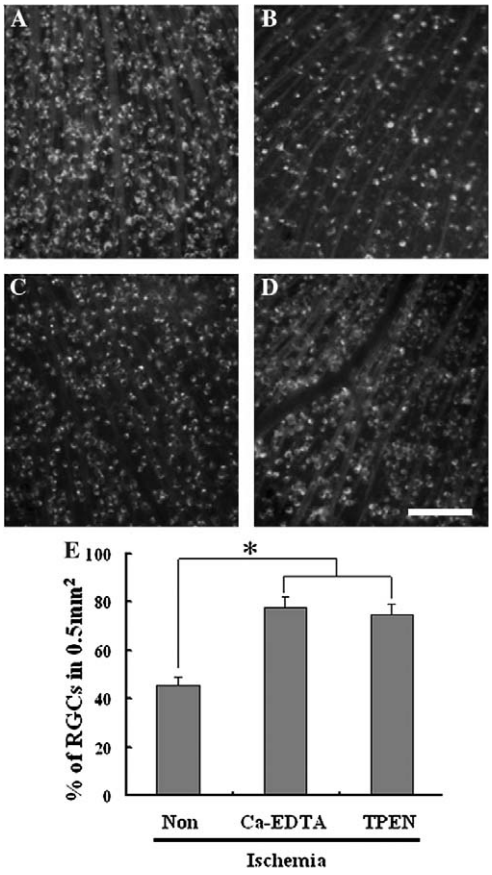

Fig. 4. Photomicrographs of retinal flat-mounts. Retinal ganglion cells were labeled retrogradely by injection of 4Di-ASP10 into the superior colliculus. (A) A normal retina, and (B) is a non-treated ischemic retina. The density of RGCs in ischemic retinas treated with Ca^{+}-EDTA (C) and TPEN (D) is higher than in non-treated ischemic retina. The ganglion cells in the temporal region were counted. (E) The viability of RGCs in the chelator treated ischemic retinas is significantly different from that of the non-treated ischemic retinas. The normal retina is set at 100%. $*p < 0.01$ by ANOVAs. Scale bar = 50 μm.

and activation of these receptor, as well as expression of p53 (Choi et al., 1989). In addition, ischemic injury induces cleavage of caspase and cause oxidative stress. In the ischemic condition, nitric oxide and lipid peroxidation are also increased and the resulting accumulation of free radicals lead to retinal cell death (Shibuki, Katai, Yodoi, Uchida, & Yoshimura, 2000; Singh et al., 2001).

According to recent studies, ischemic injury is followed by movement of zinc through NMDA channels, voltage-gated calcium channels and Ca^{2+}-A/K channels into the post-synaptic neurons where it accumulates. These movements of zinc have been confirmed in kainate-induced trauma models and ischemic models, both in vivo and in vitro (Marin et al., 2000; Sensi, Yin, Carriedo, Rao, & Weiss, 1999; Ugarte & Osborne, 2001). The accumulation of zinc has been reported to induce cell death either by reducing mitochondrial potential or increasing the concentration of reactive oxygen species, and cell death is blocked by superoxide dismutase (SOD)/catalase (Jiang, Sullivan, Sensi, Steward, & Weiss, 2001; Pong, Rong, Doctrow, & Baudry, 2002; Sheline, Behrens, & Choi, 2000; Weiss et al., 2000). Zinc-induced cell death involves some types of intra cellular signaling events such as cleavage of PARP, modulation of caspase, and depletion of glutathione (GSH) which reduces oxidative stress in neurons. In addition, zinc-induced cell death is blocked by treatment with GSH and pyruvate (Chen & Liao, 2003; Kim & Koh, 2002; Marini et al., 2001). On the other hand, zinc is reported have a protective effect as a free radical scavenger (Chimienti, Seve, Richard, Mathieu, & Favier, 2001; Dominguez, Blasco-Ibanez, Crespo, Marques-Mari, & Martinez-Guijarro, 2003). It has been proposed that synaptic zinc plays the role of an anti-cell death ion in the case of neuronal damage, in vitro and in vivo. According to these reports, chelation of zinc increases neuronal degeneration in response to excitotoxic reagents, and addition of a low concentration of zinc reduced excitotoxic cell death. Moreover, depletion of zinc results in neuronal degeneration in the normal. Therefore, zinc appears to have important bioactivity in both the neuronal degeneration and the normal condition.

Increased cyclooxygenase-2 (COX-2) level induces inflammation and cell death in ischemia. COX-2 expression was induced by neuronal ischemia and glutamate or glutamate agonists (NMDA and kinate) (Consilvio, Vincent, & Feldman, 2004; Hewett et al., 2000). In the retina, COX-2 is expressed in ischemic conditions such as glaucoma, retinal neovascularization and retinal proliferative retinopathy (Sennlaub et al., 2003; Yuan & Neufeld, 2001). It was detected in retinal ganglion cells and amacrine cells after ischemia. (Ju et al., 2003).

We confirmed above that zinc accumulation was confirmed in the IPL and INL following retinal ischemia, and that areas of zinc accumulations co-localize with degenerating cells in the ischemic retina (Fig. 1). In addition, we detected a role of zinc accumulation in retinal degeneration (Figs. 2–4). Our results indicate that inhibiting the intracellular accumulation of zinc tends to inhibit ischemia-mediated neuronal degeneration.

We used the cell-permeable zinquin ethyl ester to detect zinc ions. This chemical is specific for zinc ions and is easy to use. The zinquin is hydrolyzed within the cells and then binds to free zinc. Zinc has also been detected in neurons of the hippocampus using zinquin (Kimber, Mahadevan, Lincoln, Ward, & Tiekink, 2000; Snitsarev et al., 2001). Ca^{2+}-EDTA and TPEN were used to inhibit of zinc translocation in this study. The Ca^{2+}-EDTA used was calcium saturated to chelate metal ions, and TPEN (*N*,*N*,*N*′,*N*′ tetrakis-(2 pyridylmethyl) ethylenediamine) has a high affinity for zinc, copper, and iron ions and a low affinity for calcium ion. Ca^{2+}-EDTA is water-soluble but TPEN is not. However, TPEN is a cell-permeable zinc ion chelator (Canzoniero et al., 2003; Frederickson et al., 2002; Shumaker, Vann, Goldberg, Allen, & Wilson, 1998). We found that Ca^{2+}-EDTA and TPEN had similar effect on retinal neuroprotectine effects.

Our findings suggest that endogenous zinc ion induces COX-2 expression in neural and retinal ischemia. However,

the relationship between the free zinc and COX-2 expression requires further study.

In conclusion, the accumulation of zinc ions in retinal neurons is one of the causes of retinal degeneration and of COX-2 expression after retinal ischemia. Over accumulation of Zn^{+} may be an indicator of degeneration of retinal neurons in abnormal conditions.

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